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Both direct and indirect effects account for the pro-inflammatory activity of enteropathogenic mycotoxins on the human intestinal epithelium: Stimulation of interleukin-8 secretion, potentiation of interleukin-1β effect and increase in the transepithelial passage of commensal bacteria

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Abstract

Mycotoxins are fungal secondary metabolites responsible of food-mediated intoxication in animals and humans. Deoxynivalenol, ochratoxin A and patulin are the best known enteropathogenic mycotoxins able to alter intestinal functions resulting in malnutrition, diarrhea, vomiting and intestinal inflammation in vivo. Although their effects on intestinal barrier and transport activities have been extensively characterized, the mechanisms responsible for their pro-inflammatory effect are still poorly understood. Here we investigated if mycotoxin-induced intestinal inflammation results from a direct and/or indirect pro-inflammatory activity of these mycotoxins on human intestinal epithelial cells, using differentiated Caco-2 cells as model and interleukin 8 (IL-8) as an indicator of intestinal inflammation. Deoxynivalenol was the only mycotoxin able to directly increase IL-8 secretion (10- to 15-fold increase). We also investigated if these mycotoxins could indirectly stimulate IL-8 secretion through: (i) a modulation of the action of pro-inflammatory molecules such as the interleukin-1beta (IL-1β), and/or (ii) an increase in the transepithelial passage of non-invasive commensal *Escherichia coli*. We found that deoxynivalenol, ochratoxin A and patulin all potentiated the effect of IL-1β on IL-8 secretion (ranging from 35% to 138% increase) and increased the transepithelial passage of commensal bacteria (ranging from 12- to 1544-fold increase). In addition to potentially exacerbate established intestinal inflammation, these mycotoxins may thus participate in the induction of sepsis and intestinal inflammation in vivo. Taken together, our results suggest that the pro-inflammatory activity of enteropathogenic mycotoxins is mediated by both direct and indirect effects.

Keywords: Enteropathogenic mycotoxin; Deoxynivalenol; Ochratoxin A; Patulin; Caco-2; Interleukin 8; Intestinal inflammation

Introduction

Mycotoxins are structurally unrelated secondary metabolites produced by fungi species belonging chiefly to three genera, i.e., *Aspergillus*, *Penicillium* and *Fusarium* (Bennett and Klich, 2003; Frisvad et al., 2006; Pitt et al., 2000). Intoxications by mycotoxins have been reported to result in alteration of biological structures and functions of various tissues and systems. Thus, mycotoxins not only affect the intestinal, hepatic and/or renal epithelia but also impact on the nervous, reproductive and immune systems (Al-Anati and Petzinger, 2006; Bondy and Pestka, 2000; Bouhet and Oswald, 2005; Campbell et al., 2004; Fuchs and Peraica, 2005; Fung and Clark, 2004; Oswald et al., 2005). In addition, carcinogenic and teratogenic effects of mycotoxins have been demonstrated (Clark and Snedeker, 2006; Pfohl-Leszkowicz and Manderville, 2007; Stark, 2005).

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Mycotoxins contaminations of food and feed stuff are thus considered to lead to economic losses and health concerns both in human and animals (Bryden, 2007; Osweiler, 2000; Pineiro, 2003).

Although all food-associated mycotoxins must initially cross the intestinal wall to cause intoxication, few mycotoxins have been reported to impact on gut epithelium (Bouhet and Oswald, 2005). To date, among the twenty principal types of mycotoxins, trichothecenes (mainly deoxynivalenol or DON), ochratoxins (mainly ochratoxin A or OTA) and patulin (PAT) have the best characterized enteropathogenic activities, altering intestinal functions and leading to malabsorption, malnutrition, diarrhea, vomiting, and/or intestinal inflammation in vivo (Hunder et al., 1991; Kanisawa et al., 1977, 1990; McKinley and Carlton, 1980a,b; McKinley et al., 1982; Kubena et al., 1984; Purchase and Theron, 1968; Rotter et al., 1996; Speijers et al., 1988; Szczech et al., 1973).

In vitro models of human intestinal epithelial cells (IECs) have been successfully used to study the effects of DON, OTA and PAT on intestinal functions. Thus, it has been shown using T84, Caco-2 and HT-29-D4 cells that DON, OTA and PAT compromise the intestinal barrier function by alterations of the tight junction complex (Kasuga et al., 1998; Lambert et al., 2007; McLaughlin et al., 2004; Mahfoud et al., 2002; Maresca et al., 2001, 2002; Sergent et al., 2006). It has also been demonstrated that DON and OTA inhibit intestinal nutrient absorption, particularly the active sodium-dependent absorption of D-glucose (Maresca et al., 2001, 2002), as observed in vivo (Hunder et al., 1991). In vitro experiments, by demonstrating mycotoxin-mediated alteration of intestinal barrier and transport functions have thus provided mechanistic explanations for the in vivo observed anti-nutritional and diarrhoeic effects of mycotoxins.

Although DON, OTA and PAT cause intestinal inflammation in vivo, the mechanism(s) responsible for this effect are poorly understood (Bouhet and Oswald, 2005). Such inflammatory activity could result from a direct stimulatory effect on the production of pro-inflammatory cytokines by IECs. Indeed, it has been shown that DON, OTA and PAT can directly stimulate cytokine production by immune cells (Al-Anati and Petzinger, 2006; Bondy and Pestka, 2000; Campbell et al., 2004), suggesting a possible similar direct effect of those mycotoxins on cytokines/interleukins production by gut epithelial cells. Interestingly, secretion of the pro-inflammatory cytokine interleukin 8 (IL-8) in response to DON has recently been reported with Caco-2 and Int407 cells (Instanes and Hetland, 2004, Moon et al., 2007).

In addition to such direct effects, mycotoxin-induced intestinal inflammation could theoretically result from indirect pro-inflammatory effects. Indeed, DON is known to potentiate the effects of pro-inflammatory stimuli such as lipopolysaccharide (Islam and Pestka, 2006; Zhou et al., 1999) and bacteria (Mbandi and Pestka, 2006) on immune cells. Moreover, mycotoxins could also indirectly cause intestinal inflammation through the opening of the tight junctions allowing the entry of luminal antigens and bacteria that are normally restricted to the gut lumen by the intestinal barrier function. This could potentially lead to tissue inflammation and possibly to invasion of commensal and pathogenic bacteria as observed in Crohn's disease (Clark et al., 2005; McKay, 1999; Kleessen et al., 2002; Martin et al., 2004; Nazli et al., 2004, 2006; Swidsinski et al., 2002). While a role for intestinal barrier defects in the mycotoxin-induced gut inflammation has been postulated by us and others (Bouhet and Oswald, 2005; Mahfoud et al., 2002; Maresca et al., 2001, 2002; Sergent et al., 2006), little experimental work has been carried out on this aspect.

In the present study, we evaluated both the direct and indirect pro-inflammatory effects of DON, OTA and PAT using differentiated Caco-2 cells as the human IEC model and interleukin 8 (IL-8) as a reliable marker of intestinal inflammation and thus of pro-inflammatory activity (Baggiolini et al., 1997; Miller and Krangel, 1992; Ruchaud-Sparagano et al., 2007; Schuerer-Maly et al., 1994).

Methods

Cell line and bacteria. Parental Caco-2 cells (generous gift of Prof. Brendan Kenny, University of Newcastle, UK) were routinely grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1% L-glutamine and 1% antibiotics (all from Invitrogen) and maintained in a 5% CO_2 incubator at 37 °C. For studying mycotoxins effects, Caco-2 cells were seeded at a density of 250,000 cells per cm^2 onto Greiner inserts (ThinCert™) (1 cm^2 area, 3 μm pore size) and let to differentiate with medium changed every 2 days (Fantini et al., 1991). After 6 to 8 days of differentiation, epithelial tightness and cellular differentiation were evaluated by measurement of the transepithelial electrical resistance (TER) using an EVOM voltohmmeter (World Precision Instruments Ltd, UK). Only tight inserts with a TER > 100 Ω cm^2 were used. The commensal *Escherichia coli* K12 was generously provided by Prof. Brendan Kenny (University of Newcastle, UK) and maintained on Luria–Bertani (LB) agar plate. Single colonies were grown overnight in Luria–Bertani (LB) broth medium with the bacterial number evaluated by measuring the optical density of the bacterial suspension at 600 nm ($OD_{600\ nm}$ of 1 corresponding to approximately 10^9 bacteria/ml as determined by colony counting after bacteria plating). Bacteria were diluted to the appropriate density in DMEM (without FCS or antibiotics) before addition onto Caco-2 monolayers.

Mycotoxin treatment. DON, OTA and PAT (Sigma) stock solutions were prepared in anhydrous ethanol and stored at −20 °C. Serial dilutions of mycotoxins were prepared in anhydrous ethanol allowing the addition of similar volume of vehicle in all experiments. Caco-2 cells seeded onto inserts were treated with the indicated concentrations of mycotoxins or equivalent volumes of ethanol (untreated cells) (1% final, volume/volume). To mimic the physiological situation, mycotoxins were added apically in 0.5 ml of FCS-free DMEM, the basolateral compartment being filled with 1 ml of DMEM supplemented with 10% FCS.

Measurement of the direct effect of mycotoxins on IL-8 secretion. To evaluate the direct effect of DON, OTA and PAT on IL-8 secretion, Caco-2 cells seeded on culture inserts were apically treated either with vehicle alone or with increasing concentrations of mycotoxins for the indicated period of time. At the end of the incubation, basolateral media were collected, centrifuged (10,000×*g* for 5 min at 4 °C) and IL-8 levels measured using a commercial ELISA kit (OptEIA, BD Biosciences, France). The effect of mycotoxins on IL-8 secretion was also measured in the presence of various inhibitors (all from Sigma) such as SB 203580 (p38 inhibitor), PD 98059 (ERK1/2 inhibitor), PDTC (NF-κB inhibitor), adenine (PKR inhibitor), cycloheximide (protein synthesis inhibitor) or actinomycin D (mRNA synthesis inhibitor). In all cases, untreated cells received the same volume of vehicle than inhibitor-treated cells. Inhibitors were added basolaterally 30 min before mycotoxin treatment and were present during incubations. Preliminary experiments showed that inhibitors and vehicle solvents (ethanol and/or DMSO) used had no effect on IL-8 secretion.

Measurement of IL-8 mRNA expression levels by real-time RT–PCR. To evaluate IL-8 mRNA levels, Caco-2 cells were seeded onto 4.5 cm^2 inserts and treated either with vehicle alone or with increasing concentrations of the mycotoxin for 6 h (preliminary experiments establishing this to be the optimal incubation time). At the end of the incubation, the cells were washed three times with sterile phosphate buffer saline (PBS), scraped in PBS and centrifuged (500×*g* for 5 min at 4 °C). Cellular pellets were frozen at −80 °C until RNA extraction. Total RNA was extracted using the Trizol Reagent (Invitrogen) according to the manufacturer's protocol. DNase I-treated RNA was spectrophotometrically quantified (260 nm) and purity assessed by the $A_{260}/A_{280\ nm}$ ratio.

Reverse transcription reaction was carried out with the high capacity cDNA archive kit (Applied Biosystems) in 100 µl reaction buffer containing 1 µg of total RNA following the manufacturer's instructions. PCR primers for IL-8 mRNA and FAM dye-labeled *Taq*Man MGB probes sets were obtained from the Applied Biosystems Assays-on-demand. These primers were specifically designed to detect and quantify cDNA sequences without detecting genomic DNA. The FAM (6-carboxy-fluorescein) was used as fluorescent reporter dye and conjugated to 5′ ends of probes to detect amplification products. The amount of FAM fluorescence in each reaction liberated by the exonuclease degradation of the *Taq*Man probe during PCR amplification was measured as a function of PCR cycle number using an ABI 7000 Prism (Applied Biosystems) (Garmy et al., 2005). Oligonucleotide primers and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems as a preoptimised mix.

PCR analysis was carried out on 96-well plates of cDNA equivalent to 5 ng of total RNA. Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were collected using the ABI PRISM 7000 SDS analytical thermal cycler (Applied Biosystems). Each sample was tested in triplicate to ensure statistical significance. The relative quantification of IL-8 gene expression was performed using the comparative C_t method (Livak and Schmittgen, 2001). The C_t value is defined as the point where a statistically significant increase in the fluorescence has occurred. The number of PCR cycles (C_t) required for the FAM intensities to exceed a threshold just above background was calculated for the test and reference reactions. In all experiments, GAPDH was used as the endogenous control. Results were analyzed in a relative quantitation study with the vehicle treated Caco-2 inserts serving as the calibrator (sample used as the basis for comparative results). Negative controls were included in the reaction plate: (i) a minus reverse transcriptase control (mock reverse transcription with all the RT–PCR reagents except the reverse transcriptase) and (ii) a minus sample control containing all the RT–PCR reagents except the RNA template. No products were synthesized in those controls.

Measurement of the effect of mycotoxins on the IL-8 secretion induced by IL-1β. Caco-2 cells were incubated with various concentrations of the indicated mycotoxin for 12 h. The cells were then washed with culture medium and treated for 3 h with 20 ng/ml of human recombinant IL-1β (Peprotech, France) added basolaterally (the concentration of IL-1β and the time of treatment having been optimized during preliminary experiments). At the end of the incubation, IL-8 secretion was measured as described above.

Measurement of the epithelial barrier function. Intestinal barrier function was evaluated by three distinct assays measuring: (i) TER; (ii) transepithelial passage of FITC-dextran (Sigma, 4 kDa); (iii) transepithelial passage of horseradish peroxidase (HRP) (Sigma, 45 kDa). In all cases, Caco-2 cells were cultured onto 3 µm pore size inserts as described above and treated for 12 h with either vehicle or mycotoxins. TER values were measured using an EVOM voltohmmeter and the apical to basal flux of tracer was measured as follows. FITC-dextran and HRP were diluted in phenol red-free Hank's balanced salt solution (HBSS) at 1 mg/ml and were added apically onto HBSS washed Caco-2 inserts (0.5 ml added), the basolateral compartment being also filled with HBSS. After 1 h at 37 °C in the CO_2 incubator, basolateral media were collected and tracer concentrations were measured. The amount of FITC-dextran was measured using a Fluoroscan Ascent spectrofluorometer (excitation at 485 nm and emission at 538 nm). The amount of HRP was measured enzymatically using known concentrations of HRP as standard and OPD as substrate. As a positive control for tight junction opening, the TER and the transepithelial passage of tracers of calcium-free treated cells (30 min incubation in FCS/calcium/magnesium-free DMEM with 2 mM EDTA added) was measured in parallel.

Measurement of the passage of commensal bacteria through Caco-2 epithelium. Transepithelial passage of non-invasive commensal *E. coli* K12 through Caco-2 inserts was measured as follows. Caco-2 cells seeded onto 3 µm pore size inserts were treated with either vehicle alone or mycotoxins for 12 h with calcium-free treated cells included as a positive control of tight junction opening. Commensal bacteria grown overnight in LB solution were diluted in DMEM without FCS or antibiotics and added apically to the insert at a IEC/bacteria ratio of 1:100 (i.e. 10^7 bacteria). After 6 h at 37 °C in the CO_2 incubator, basolateral media were collected and serially diluted in DMEM without FCS or antibiotics. 20 µl of each dilution were plated onto LB agar Petri dishes, incubated overnight (37 °C) before colonies counting, with the number of bacteria present in 1 ml expressed as colony-forming unit (cfu) per ml.

Measurement of the effect of mycotoxins on IL-8 secretion induced by commensal bacteria. Caco-2 cells seeded onto 3 µm pore size inserts were treated with vehicle or mycotoxins for 12 h to compromise tight junctions with calcium-free treated cells used as a positive control of tight junction opening. As above, commensal *E. coli* K12 were grown overnight in LB solution and diluted in DMEM without FCS or antibiotics before apical or basolateral addition to Caco-2 inserts at a IEC/bacteria ratio of 1:100 (i.e. 10^7 bacteria). After 6 h, basolateral media were collected, centrifuged (10,000×*g* for 5 min at 4 °C) and levels of secreted IL-8 determined by ELISA.

Statistical analysis. All experiments were conducted in triplicate. Results were expressed as means ± standard deviation (S.D.) ANOVA analysis was used to address the significance of differences between mean values with significance set at $p \leq 0.05$.

Results

Only DON possesses direct effect on IL-8 secretion by human IEC

First, we tested the direct effect of increasing concentrations of DON, OTA and PAT on IL-8 secretion from differentiated Caco-2 cells after 12 h of treatment (Fig. 1). The only mycotoxin possessing a direct effect was DON with a statistically

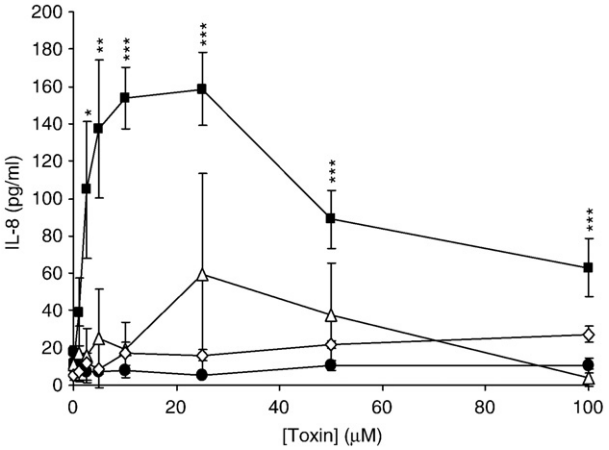

Fig. 1. Dose-dependent direct effect of DON, OTA and PAT on IL-8 secretion by Caco-2 cells. Caco-2 cells seeded onto inserts were left untreated or apically treated with increasing concentrations of DON (closed squares), OTA (closed circles), PAT (opened triangles) or CHX (opened diamonds) using the same volume of vehicle (ethanol, 1% final vol./vol.) as outlined in Methods. After 12 h of incubation, the levels of secreted IL-8 were measured (ELISA). Each point represents the mean of six independent experiments ± S.D. ($n=6$). Statistical significance was assessed by ANOVA with *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to untreated cells.

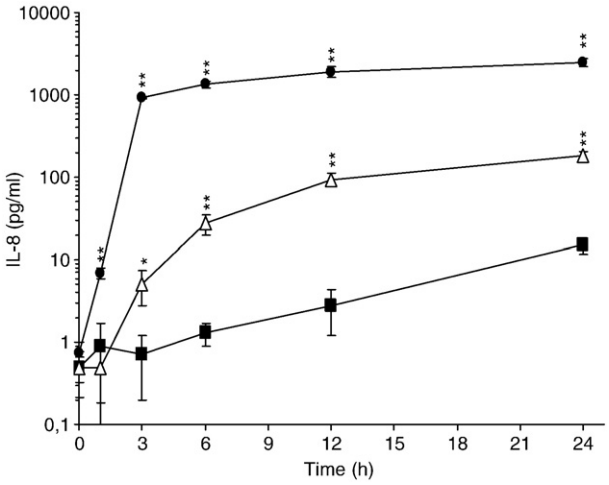

Fig. 2. Time-dependent direct effect of DON and IL-1β on IL-8 secretion by Caco-2 cells. Caco-2 cells seeded onto inserts were left untreated (closed squares) or treated with 10 μM of DON added apically (opened triangles) or 20 ng/ml of IL-1β added basolaterally (closed circles) for the indicated time period prior to measuring IL-8 secretion levels. Each point represents the mean of three independent experiments±S.D. ($n=3$). Statistical significance was assessed by ANOVA with *$p<0.01$ and **$p<0.001$ compared to untreated cells at the same time.

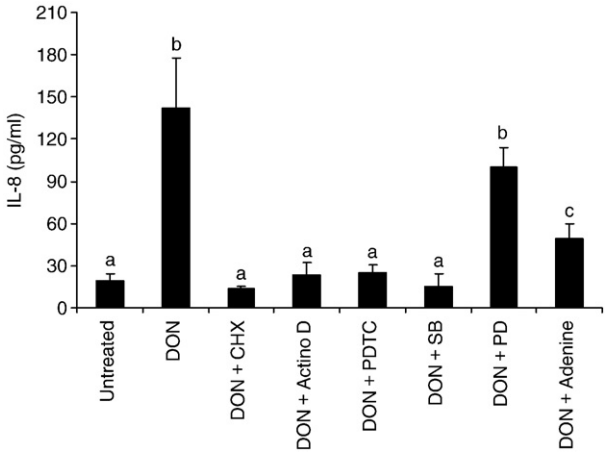

Fig. 3. Implicating signalling pathway(s) involved in the direct effect of DON on IL-8 secretion by Caco-2 cells. Caco-2 cells seeded onto inserts were left untreated or pre-incubated basolaterally for 30 min with various inhibitors: 300 μM cycloheximide (CHX); 10 μg/ml actinomycin D (Actino D); 100 μM PDTC; 10 μM SB 203580 (SB); 50 μM PD 98059 (PD) or 2 mM adenine, prior to the apical addition of DON (10 μM). The inhibitors were present during the 12-h incubation prior to measuring IL-8 secretion levels. Each bar represents the mean of three independent experiments±S.D. ($n=3$). Statistical significance was assessed by ANOVA. Bars without a common letter differ by at least $p<0.05$.

significant 10-fold increase in IL-8 secretion for concentrations of toxin equal to or greater than 2.5 μM ($p<0.05$). Importantly, the effect of DON on IL-8 secretion was found to be biphasic with (i) an initial increase for concentrations equal to or less than 25 μM (15-fold increase, $p<0.001$) and (ii) a decrease in IL-8 secretion at concentrations of 50 μM or more, with the level of secreted IL-8 at these doses remaining 6 to 8 times higher than that of untreated cells ($p<0.001$). In contrast to DON, neither OTA nor PAT had a significant affect on IL-8 secretion, though PAT exhibited some inconsistent activity. This effect of DON on IL-8 secretion was unrelated to its capacity to inhibit protein synthesis, as the protein synthesis inhibitor cycloheximide (CHX) failed to induce IL-8 secretion (Fig. 1).

Next, we examined the kinetics of IL-8 secretion with an optimal concentration of DON (i.e. 10 μM) compared to that mediated by a recognised inducer of IL-8 secretion, IL-1β (Fig. 2). At the physiologically relevant dose of 20 ng/ml (Al-Sadi and Ma, 2007; Iwamoto et al., 1989) IL-1β induced a strong IL-8 secretory response, whereby a rapid increase occurred during the first 3 h followed by a plateau. By contrast IL-8 secretion induced by DON was weaker (approximately 10 times) and the kinetics were different, with a lag period (between 0 and 1 h), followed by a gradual increase (to 12 h) followed by a plateau.

Real-time RT–PCR evaluation of DON effect on IL-8 mRNAs

We next studied the effect of DON on the IL-8 mRNA levels using real-time RT–PCR (Table 1). We found that DON caused a dose-dependent increase in IL-8 mRNA levels that remained high at 100 μM, in marked contrast to the corresponding IL-8

Table 1
Relative quantification of IL-8 mRNA in Caco-2 cells upon DON stimulation

Sample	Target IL-8 C_t [a]	Endogenous control GAPDH C_t	ΔC_t [b] IL-8 − GAPDH	$-\Delta\Delta C_t$ [c] $-(\Delta C_{t\ \text{IL-8}} - \Delta C_{t\ \text{calibrator}})$	$2^{-\Delta\Delta C_t}$ [d] IL-8 relative to untreated cells
Untreated cells (calibrator)	33.21±0.70	19.30±0.96	13.90±0.35	0.00±0.35	1.00±0.69 (0.73 to 1.27)
DON 1 μM	32.65±0.24	20.21±0.22	12.44±0.41*	0.56±0.41	2.43±0.87 (1.91 to 3.44)
DON 10 μM	31.20±0.09**	20.87±0.49	10.24±0.43**	2.00±0.43**	11.20±1.00** (10.49 to 11.91)
DON 100 μM	30.31±0.44***	19.91±1.13	10.40±0.77**	2.89±0.77*	12.47±5.81* (5.88 to 16.86)

The data are expressed as the mean±S.D. of three independent experiments ($n=3$) each performed in duplicate with *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to untreated cells.

[a] The threshold cycle (C_t value) is the cycle at which the system begins to detect the increase in the signal associated with an exponential growth of PCR products during the log-linear phase.

[b] ΔC_t=Target C_t−GAPDH C_t.

[c] $-\Delta\Delta C_t=-(\Delta C_t$ target$-\Delta C_t$ calibrator). In these experiments, untreated Caco-2 cells are used as internal calibrator. The calibrator is the 1× sample and all other quantities are expressed as an n-fold difference relative to the calibrator.

[d] The range given for IL-8 mRNA from DON-treated relative to untreated Caco-2 cells is determined by evaluating the expression: $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t+s$ and $\Delta\Delta C_t-s$, where s is the standard deviation of the $\Delta\Delta C_t$ value.

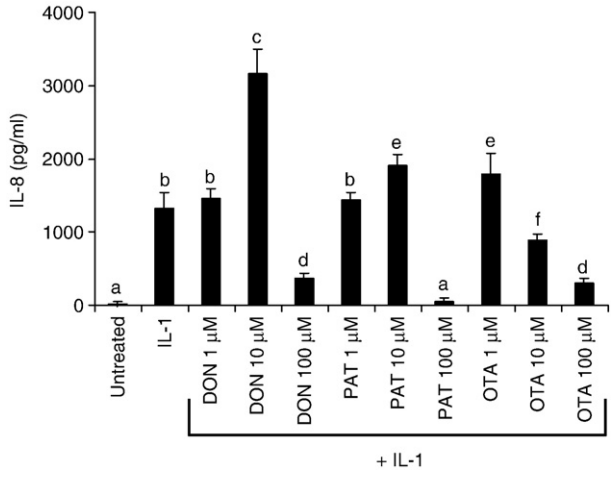

Fig. 4. Impact of DON, OTA and PAT on the IL-8 secretion induced by treatment of Caco-2 cells with IL-1β. Caco-2 cells seeded onto inserts were either left untreated or treated with the indicated concentration of mycotoxin for 12 h. IL-1β was then basolaterally added at 20 ng/ml for 3 h after which IL-8-secreted levels were measured. Each bar represents the mean of three independent experiments±S.D. (n=3). Statistical significance was assessed by ANOVA. Bars without a common letter differ by at least $p<0.05$.

secretion levels. Moreover, the effect of DON on IL-8 mRNAs was not biphasic, as 10 and 100 μM of DON induced statistically similar increases in mRNA levels (11.20±1.00 and 12.47±5.81 times increase in relative IL-8 mRNAs expression ($2^{-\Delta\Delta C_t}$) for 10 and 100 μM of DON, respectively, p=0.7).

DON-induced IL-8 secretion by human IEC depends on PKR, p38 and NF-κB

In an attempt to characterize the signal pathway(s) involved in the direct effect of DON on IL-8 secretion, the effect of an optimal dose of DON, i.e. 10 μM was tested in the presence of various inhibitors (Fig. 3). We found that inhibitors of mRNA and protein synthesis (actinomycin D and cycloheximide, respectively) completely inhibited DON-mediated IL-8 secretion (96±7% and 104±1% inhibition, $p<0.001$). Similarly, PDTC, an inhibitor of the transcriptional activator NF-κB significantly suppressed IL-8 secretion levels (95±4% inhibition, $p<0.001$). The MAP kinase p38 inhibitor SB 203580 significantly abolished DON effect (102±7% inhibition, $p<0.001$), while the ERK1/2 inhibitor PD 98059 only caused a weak and non significant inhibition (34±11% inhibition, p=0.13). Finally, the protein kinase R (PKR) inhibitor adenine was tested and was found able to significantly inhibit the DON effect (75±8% inhibition, $p<0.05$).

DON, OTA and PAT affect the IL-8 secretion caused by treatment of human IEC with IL-1β

Next, we investigated if mycotoxin treatment would affect the response of Caco-2 cells to IL-1β. We found (Fig. 4) that 100 μM of DON, OTA or PAT all had a statistically significant inhibitory impact on IL-1β-induced IL-8 secretion (72±5%, 96±4% and 77±5% inhibition, respectively ($p<0.001$)). However, lower concentrations of mycotoxins had the opposite effect with 1 μM of OTA and 10 μM of DON or PAT causing a 35±8%, 138±25% and 44±10%, respectively (at least $p<0.05$), in IL-1β-induced IL-8 secretion.

DON, OTA and PAT affect intestinal permeability

When we evaluated the effect of mycotoxins on intestinal permeability through TER measurements (Table 2), we found, as previously published by us and others (Kasuga et al., 1998; McLaughlin et al., 2004; Mahfoud et al., 2002; Maresca et al., 2001, 2002; Sergent et al., 2006) that DON, OTA and PAT induced a dose- and time-dependent drop in TER, with only high doses known to be cytotoxic causing significant effects. Next, we investigated the effect of mycotoxins on intestinal permeability through tracer's flux measurement relative to the TER measurement data. In these experiments, calcium-free medium treated cells were used as positive control for tight junction opening. As expected, the absence of calcium resulted in a major decrease in TER (71±5% decrease, $p<0.001$) (Fig. 5A) and a significant increase in FITC-dextran and HRP flux (77±5- and 33±0.7-fold increase, $p<0.001$) (Figs. 5B and C). Similarly, increase in fluxes of FITC-dextran and HRP were only observed with concentrations of mycotoxins that caused a significant drop in TER, i.e. 100 μM (Figs. 5B and C).

Table 2
Dose and time-dependent effects of DON, OTA and PAT on the TER of Caco-2 cells

Time (h)	0	1	3	6	12	24
Untreated	144±12	142±8	139±10	137±4	142±7	158±11
DON 1 μM	142±8	145±6	143±7	139±1	156±16	158±9
DON 10 μM	140±6	134±12	145 ±9	139±3	153±3	74±9***
DON 100 μM	137±9	143±6	124±7*	118±2**	75±8***	16±5***
PAT 1μM	143±12	144±14	134±7	140±4	160±10	159±15
PAT 10 μM	145±4	140±5	142±9	145±8	156±6	158±9
PAT 100 μM	144±9	143±12	109±11*	57±9***	53±6***	18±7***
OTA 1 μM	139±4	152±24	155±12	156±10	147±9	153±8
OTA 10 μM	134±7	147±14	134±13	141±10	146±8	133±12
OTA 100 μM	145±3	141±8	145±7	144±6	121±9**	48±8***

TER values of either not treated or treated Caco-2 cells were measured using an EVOM voltohmmeter as explained in Methods. The data are expressed as the mean± S.D. (n=3) of three independent experiments with *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to untreated cells.

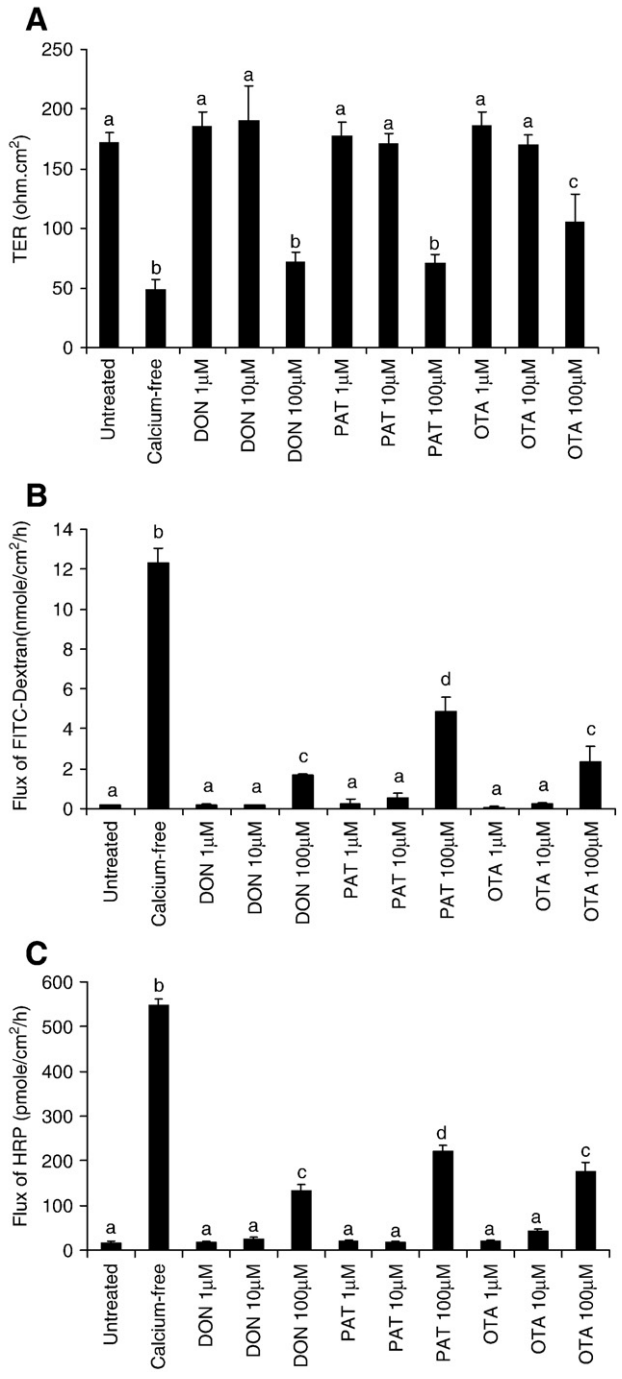

Fig. 5. Impact of DON, OTA and PAT on the intestinal barrier function in term of transepithelial electrical resistance and transepithelial passage of FITC-dextran and HRP. Caco-2 cells seeded onto inserts were either left untreated or treated with the indicated concentration of mycotoxin for 12 h, with cells incubated in calcium-free media used as positive control for tight junction opening. TER (A) and paracellular flux of FITC-dextran (4 kDa) (B) and HRP (C) were measured. Each bar represents the mean of three independent experiments ± S.D. ($n=3$) with statistical significance assessed by ANOVA. Bars without a common letter differ by at least $p<0.05$.

DON, OTA and PAT increase the passage of commensal bacteria through the intestinal epithelium

We also assessed the passage of commensal (non-invasive) *E. coli* through Caco-2 cells treated for 12 h with 1, 10 or 100 µM of mycotoxins (Fig. 6). As expected, we found that treatments inducing a drop in TER and increase in the flux of tracer, i.e. 100 µM mycotoxins or calcium-free treated cells, led to a significant increase in the passage of bacteria (at least $p<0.05$). Surprisingly, lower concentrations of toxin (i.e. 1 µM PAT or OTA and 10 µM DON, OTA or PAT), that do not alter barrier function, in terms of TER or flux measurement (Fig. 5), induced a significant increase in bacterial passage (with at least $p<0.05$).

The increase in bacterial passage caused by DON and PAT results in an indirect stimulation of the IL-8 secretion by human IEC

We measured the impact of mycotoxin treatment on IL-8 secretion induced by commensal *E. coli* (Fig. 7). Stimulatory effect of bacteria on IL-8 secretion was found polarized in untreated cells. Indeed, apically added bacteria (Fig. 7A) caused a weaker IL-8 secretion compared to basolateral exposure (Fig 7B) (10 ± 0.6- and 53 ± 6-fold increase, respectively, in IL-8 secretion compared to untreated cells without K12 added ($p<0.001$)). Next, we investigated the impact of calcium-free condition on bacterial effect. Calcium removal resulted by itself in IL-8 secretion (46 ± 5-fold increase compared to untreated cells without K12 added ($p<0.001$)). Although quite unexpected, such pro-inflammatory effect caused by calcium-removal has been, however, already described in human bronchial epithelial cells (Shibata et al., 1996). In addition to this direct effect, tight junction opening caused by calcium removal also resulted in an increase in the effect of apically added bacteria on IL-8 secretion (1.6 ± 0.06-fold increase compared to calcium-free treated cells without K12 apically added, $p<0.05$).

Concerning the action of mycotoxins on the effect of apically added K12 (Fig. 7A), we found that treatment of Caco-2 cells

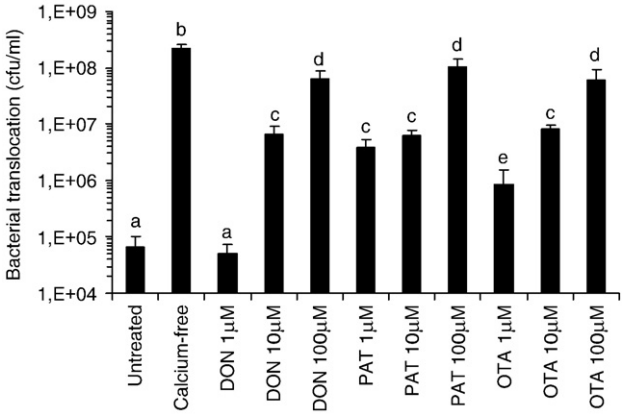

Fig. 6. Impact of DON, OTA and PAT on the transepithelial passage of non-invasive commensal *E. coli.* Caco-2 cells seeded onto inserts were either left untreated or treated with the indicated concentration of mycotoxin for 12 h, with cells incubated with calcium-free media used as positive control for tight junction opening, prior to measuring transepithelial passage of K12 *E. coli*. Each bar represents the mean of three independent experiments ± S.D. ($n=3$), with statistical significance assessed by ANOVA. Bars without a common letter differ by at least $p<0.05$.

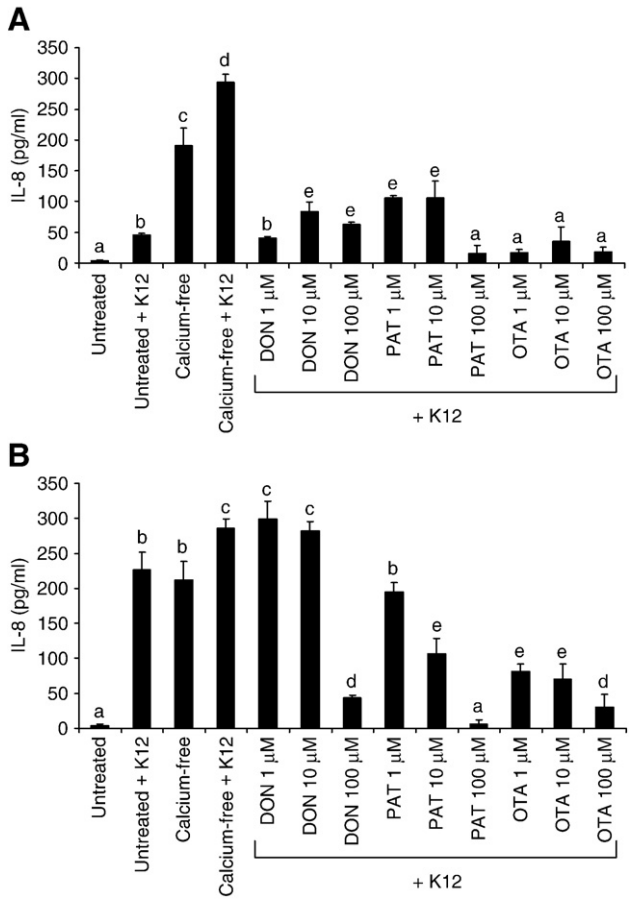

Fig. 7. Impact of DON, OTA and PAT on the IL-8 secretion induced by non-invasive commensal *E. coli*. Caco-2 cells seeded onto inserts were either left untreated or treated with the indicated concentration of mycotoxin for 12 h, with cells incubated in calcium-free media acting as a positive control for tight junction opening. K12 were then added apically (A) or basolaterally (B) at a IEC/bacteria ratio of 1:100 for 6 h prior to measuring IL-8-secreted levels. Each bar represents the mean of three independent experiments ± S.D. ($n=3$), with statistical significance assessed by ANOVA. Bars without a common letter differ with at least $p<0.05$.

with 10 or 100 μM of DON and 1 or 10 μM of PAT resulted in a significant increase in the effect of the bacteria (1.8±0.3-, 1.35±0.08-, 2.3±0.09- and 2.4±0.6-fold increase compared to untreated cells with K12 apically added, $p<0.05$). On the opposite, treatment with 100 μM of PAT or 1, 10 or 100 μM of OTA all resulted in a significant decrease in the effect of apically added bacteria ($p<0.05$). Finally, we investigated the impact of the mycotoxins on the ability of basolaterally added K12 to induce IL-8 secretion (Fig. 7B). We found that 100 μM of DON, 10 or 100 μM of PAT and 1, 10 or 100 μM of OTA all had a significant impact on the ability of commensal *E. coli* K12 to induce IL-8 secretion ($p<0.05$). In contrast, lower concentrations of DON (1 and 10 μM) had a significant potentiating effect (1.32±0.16- and 1.24±0.05-fold increases in IL-8 levels compared to mycotoxin-untreated basolaterally infected cells, $p<0.05$).

Discussion

In the present study, we investigated the ability of three important enteropathogenic mycotoxins (i.e. DON, OTA, and PAT) to induce the secretion of the pro-inflammatory cytokine IL-8 from human intestinal epithelial cells by direct and/or indirect mechanisms.

Our experiments revealed that, of the three mycotoxins tested, the only one that directly stimulated IL-8 secretion from human IEC is DON, in accordance with other studies (Instanes and Hetland, 2004, Moon et al., 2007). However, we have now demonstrated that this is due to a direct stimulatory effect that is: (i) independent of protein synthesis inhibitory functions of mycotoxins, (ii) dependent on the ribotoxic-associated activation of (a) protein kinase R (PKR), (b) the transcriptional activator NF-κB and (c) the MAP kinase p38 – the latter consistent with studies with immune cells (Pestka et al., 2004; Zhou et al., 2003a). It should be noted that in marked contrast to our results, DON induced IL-8 secretion in the human IEC model Int407 was dependent on the MAP kinase ERK1/2 but not p38 (Moon et al., 2007). We have no formal explanations for this discrepancy, although the differentiation nature of the Caco-2 versus undifferentiated Int407 cells may be involved.

We also described a novel biphasic nature for the DON effect on IL-8 secretion. DON first increased IL-8 secretion with a statistically significant effect for concentrations superior or equal to 2.5 μM (10 times increase) and a maximal stimulation observed at 25 μM of DON (15-fold increase). The initial increase was followed by a decrease in the secretion of IL-8 for concentrations of toxin superior or equal to 50 μM, though the reduced IL-8-secreted levels remained 6- to 8-fold greater than untreated control cells. Using IL-1β stimulation, we demonstrated that the second part of the biphasic effect of DON is due to an active inhibition of the IL-8 secretion at high concentrations of DON. Quantitative RT–PCR analysis suggested that this inhibition of IL-8 secretion by DON relates to IL-8 protein synthesis/stability since IL-8 mRNA levels are similar at 10 and 100 μM of DON.

Experiments with IL-1β also showed that high concentrations of DON, OTA and PAT all inhibited this response, presumably relating to their well-defined cellular toxicity to IEC cells (Mahfoud et al., 2002; Maresca et al., 2001, 2002). However, lower concentrations of mycotoxins, that are more likely to occur during natural contaminations, unexpectedly potentiated the IL-1β-induced IL-8 secretion, suggesting that ingestion of DON, OTA or PAT could exacerbate existing established intestinal inflammation.

In addition to direct pro-inflammatory effect and potentiation of established inflammation, mycotoxins could also theoretically cause intestinal inflammation indirectly through alteration of the intestinal barrier function. It has been previously established that the detection of bacteria by IEC, which induces IL-8 secretion, is exclusively mediated through the interaction of bacterial flagella with the cellular Toll-like receptor 5 (TLR-5) (Bambou et al., 2004; Ruchaud-Sparagano et al., 2007; Zhou et al., 2003b). As a consequence of polarized basolateral expression of TLR-5, IEC are much more efficient at detecting bacteria when bacteria are present in the basolateral compartment [previous published studies (Bambou et al., 2004; Ruchaud-Sparagano et al., 2007; Zhou et al., 2003b) and our results]. An indirect pro-inflammatory effect of mycotoxins could thus result from an alteration of the intestinal barrier function allowing the

transepithelial passage of non-invasive commensal bacteria, although direct evidence of such effect has been absent until now.

Our data support previous studies (Kasuga et al., 1998; McLaughlin et al., 2004; Mahfoud et al., 2002; Maresca et al., 2001, 2002; Sergent et al., 2006) showing that only long term treatment with cytotoxic doses of mycotoxins (i.e. around 100 μM) compromise tight junctions as indicated by a drop in TER and an increase in the passage of paracellular tracers. As expected, we found that these high doses of mycotoxins allowed the transepithelial passage of apically added non-invasive commensal bacteria. Surprisingly, we also observed a significant increase in bacterial passage at lower toxin concentrations that do not compromise barrier function (as assessed by TER and flux of tracers). Thus, it seems plausible that mycotoxin treatment causes an increase in the transcellular translocation of commensal bacteria through Caco-2 cells, as previously observed during ATP depletion or interferon γ treatment of human IECs (Clark et al., 2005; Nazli et al., 2004, 2006).

Although not yet fully understood, such increase in the bacterial passage through IEC after DON, OTA or PAT treatment could have major implications for human health in term of sepsis and inflammation. Indeed, inflammatory bowel diseases (IBD), such as Crohn's disease, are generally associated with the presence of adherent-invasive bacteria (Boudeau et al., 1999; Sasaki et al., 2007) but although with an unexplained increase in the passage of non-invasive commensal bacteria through the gut epithelium (Clark et al., 2005; McKay, 1999; Kleessen et al., 2002; Martin et al., 2004; Nazli et al., 2004, 2006; Swidsinski et al., 2002). An attractive hypothesis would be that, at least in some cases, ingestion of food contaminated with mycotoxins could be involved in inducing IBD. In agreement with this, we demonstrated that the increase in bacterial passage linked to DON and PAT treatment was associated with increased IL-8 secretion levels.

Our data also showed that, although OTA increased the passage of bacteria, it did not potentiate their effect on IL-8 secretion. Studies of the effect of mycotoxins on the IL-8 secretion induced by basolaterally added bacteria revealed that OTA in fact inhibited the detection of bacteria by IEC. Such inhibition, in addition to the increase in bacterial passage caused by OTA treatment, potentially explains the hyper-susceptibility of animals to intestinal bacterial infection observed after OTA ingestion (Fukata et al., 1996). Indeed, it should be noted that a similar conclusion was drawn for fumonisin B1 (FB1), a mycotoxin that opens tight junctions and inhibits IL-8 secretion from porcine IEC (Bouhet et al., 2006). Like OTA, high doses of DON or PAT also increased bacterial passage and inhibited their detection by IEC, suggesting a potential increase in the susceptibility to intestinal bacterial invasion during DON or PAT intoxication. We also found, as reported with studies on immune cells (Mbandi and Pestka, 2006), that 1 and 10 μM DON potentiates the effect of basolaterally added bacteria on IL-8 secretion by human IEC. The mechanism(s) underlying such synergy between DON and bacteria remain to be defined.

Taken together, our in vitro data suggest that the pro-inflammatory activity observed during in vivo intoxication with three major enteropathogenic mycotoxins, i.e. DON, OTA and PAT, is mediated by both direct and indirect effects.

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